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Spirostaphylotrichin W, a spirocyclic γ-lactam isolated from liquid culture of *Pyrenophora semeniperda*, a potential mycoherbicide for cheatgrass (*Bromus tectorum*) biocontrol

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ABSTRACT

A novel spirocyclic γ-lactam, named spirostaphylotrichin W (**1**), was isolated together with the well known and closely related spirostaphylotrichins A, C, D, R and V, as well as triticone E, from the liquid cultures of *Pyrenophora semeniperda* (anamorph: *Drechslera*), a seed pathogen proposed for cheatgrass (*Bromus tectorum*) biocontrol. Spirostaphylotrichin W was characterized as (3*S**,4*S**,5*S**,6*S**,9*Z*,10*Z*)-4,6-dihydroxy-2,3-dimethoxy-3-methyl-10-propyliden-2-azaspiro [4.5]dec-8-ene-1,7-dione, by spectroscopic and chemical methods. The relative stereochemistry of spirostaphylotrichin W was assigned using NOESY experiments and in comparison to those of spirostaphylotrichin V (**2**) and triticone E (**6**). In fact, the relative stereochemistry at C-3 was the same of that of **2**, while that at C-4 and C-6 was inverted in respect to that reported, respectively, for **2** and **6**. In a *B. tectorum* coleoptile bioassay at concentration of 10^{-3}, spirostaphylotrichin A proved to be the most active compound, followed by spirostaphylotrichins C and D. Spirostaphylotrichin W and V showed mild toxicity while spirostaphylotrichin R and triticone E were not active. When tested on host and non-host plants by leaf puncture bioassay, spirostaphylotrichins A, C and D caused the appearance of necrotic spots while the other compounds were inactive.

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1. Introduction

Cheatgrass (*Bromus tectorum*) is an exotic winter annual grass weed that has invaded millions of hectares of rangeland in western North America. Several fungal pathogens have been investigated as potential biocontrol agents for this weed, and the seed pathogen *Pyrenophora semeniperda* is one of the most promising.[1] It was discovered in strain trials with this pathogen that mycelial growth rate was negatively correlated with virulence on non-dormant cheatgrass seeds.[2] It was hypothesized that this apparent trade-off between virulence and growth was due to the production of metabolically expensive toxins needed to quickly disable germinating seeds.[2] This prompted studies of the phytotoxic metabolites produced by this fungus. It was previously determined that an Australian strain of *P. semeniperda* grown on wheat seeds produced large quantities of the phytotoxic cytochalasin B, along with cytochalasin A, cytochalasin F, deoxaphomin, and three novel cytochalasins, Z1, Z2, and Z3.[3] Recently, a rapid and sensitive HPLC method was developed for quantification of cytochalasin B in solid wheat seed and liquid PDB (potato dextrose broth) cultures in order to evaluate its use in virulence screening.[4]

All strains produced large quantities of cytochalasin B in wheat seed culture, but none produced this compound in PDB culture. The culture filtrates, however, were often toxic to germinating cheatgrass seeds, a result also found in other studies of this fungus.[5] Preliminary TLC investigations of the culture filtrate organic extracts showed the presence of metabolites potentially belonging to a different class of natural compounds. This paper reports on the isolation, identification and biological characterization of five already known spirostaphylotrichins, A, C, D, R and V, and triticone E from these culture filtrate extracts, as well as the structure and biological characterization of the new spirostaphylotrichin W.

2. Results and discussion

The strains TMC10-34 and WRK10-22 of *P. semeniperda* were cultured in PDB, and the organic extracts from their filtrates were fractionated by column chromatography and TLC (see Experimental

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section) yielding spirostaphylotrichins C, D, R, V and triticone E (**3**, **4**, **7**, **2** and **6**, Scheme 1) from the strain WRK10-22 and spiro-staphylotrichin A (**5**, Scheme 1) from the strain TMC10-34.
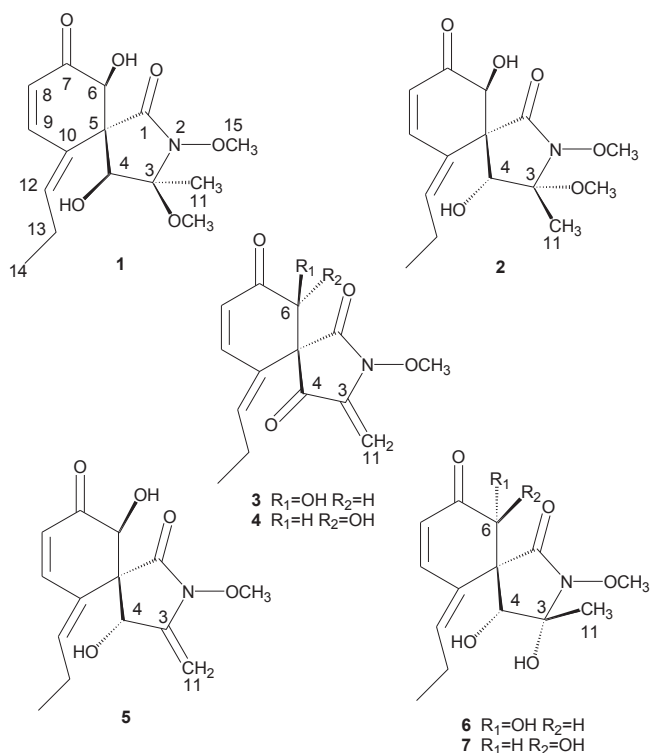
Scheme 1. Structures of spirostaphylotrichins W, V, C, D, A and R (**1–5** and **7**) and triticone E (**6**).

The structures of these compounds were identified by comparing their ^{1}H NMR spectra with the data reported in literature for spirostaphylotrichins C and D (**3** and **4**),[6] for triticone E and spiro-staphylotrichin R (**6** and **7**),[6,7] and for spirostaphylotrichin V (**2**).[7] The physical and spectroscopic data for spirostaphylotrichin A (**5**) (OR, ^{1}H and ^{13}C NMR, IR and UV) were compared to all the data previously reported for this compound.[6] The structure of **5** was also supported by COSY, HSQC, HMBC, and NOESY spectra and by the data from HRESIMS, which showed the dimeric sodium form [2M+Na]+, the sodiated cluster [M+Na]+ and the pseudomolecular ion [M+H]+ at m/z 581.2124, 302.0994 and 280.1176, respectively. The identification of **2**, **3**, **4**, **6** and **7** were also confirmed by ESI and/or APCIMS. The compounds **3** and **4**,[6] as well as **6** and **7**,[6,7] were isolated as a mixture. The chromatographic separation of these latter compounds was not possible due to their interconversion as also previously observed.[6,8] The most plausible mechanism to explain this interconversion is a retro-aldol type reaction, as first reported by Sugawara et al. (1988)[8] and then by Hallock et al. (1993).[6]

An additional metabolite was isolated from strain WRK10-22. It showed a molecular formula of $C_{15}H_{21}NO_6$ as deduced by its HRE-SIMS, consistent with six double bond equivalents.

These results and the preliminary investigations of its ^{1}H and ^{13}C spectra (Table 1) showed that it is closely related to spirophylotrichins and being new as described below, was named spiro-staphylotrichin W (**1**, Scheme 1). The spirostaphylotrichin closest to **1** appeared to be spirostaphylotrichin V (**2**). In fact, the IR spectrum of **1** showed hydroxyl, α-β unsaturated and amidic carbonyl groups,[9] while the UV spectrum showed absorption maxima at 290 and 202, typical of an extended conjugated α,β-unsaturated carbonyl group.[10] Its ^{1}H NMR spectrum (Table 1) showed two doublets (J=9.9 Hz) at δ 7.11 (H-9) and 5.94 (H-8), typical of a *cis*-disubstituted double bond

of a cyclohexenone ring, which appeared coupled in the COSY spectrum.[11] The same spectrum also showed the signal of a 1-propenyl side chain with the olefinic proton (H-12) resonating as a triplet (J=7.4 Hz) at δ 6.12. H-12 in the COSY spectrum, coupled with both protons of the adjacent methylene group (H2C-13), observed as two multiplets at δ 2.24 and 2.15. The latter, in turn, coupled with the terminal methyl group (Me-14) appearing as a triplet (J=7.4 Hz) at δ 1.05. The signals of the protons of two hydroxylated secondary carbons (HC-6 and HC-4) resonated as two doublets (J=1.7 and 11.5 Hz) at δ 4.75 and 4.12, being coupled with the geminal hydroxyl groups appearing as two doublets at δ 3.80 (HO-6) and 2.66 (HO-4), respectively.[10] The singlets of N−OMe, OMe and Me-11 groups resonated at δ 3.94, 3.51, and 1.69, respectively. The correlations observed in the HSQC spectrum[11] allowed assignment of the carbons observed in the ^{13}C NMR spectrum (Table 1) at δ 153.2, 120.5, 149.5, 23.4, 13.2, 73.5, 70.1, 64.5, 52.7 and 17.5 to C-9, C-8, C-12, C-13, C-14, C-6, C-4, C-15, OMe and C-11, respectively. The ^{13}C NMR spectrum also showed the signals of α,β-unsaturated (C-7) and amidic (C-1) carbonyl groups at typical chemical shift values of δ 196.8 and 167.6.[12] The same spectrum also showed the presence of two quaternary carbons (C-5 and C-3) at δ 56.6 and 88.8. C-5 appeared to be the spirocarbon linking the cyclohexenone and the γ-lactam rings. In fact, in the HMBC spectrum[11] C-5 coupled with H-12, H-9, H-6, H-4, HO-6 and Me-11 while C-3, which is the γ-carbon of the γ-lactam ring, coupled with H-4, HO-4, MeO and Me-11. These latter couplings also allowed localization of both Me-11 and O−Me at C-3 while the 1-propenyl side chain was localized at C-10 by the couplings observed between C-10 with H2-13, C-9 with H-12 and H2-13 and C-8 with H-12. These results and the all the other couplings observed in the HMBC spectrum allowed assignment of the chemical shifts to all the carbons and the corresponding protons (Table 1) and to formulate spirostaphylotrichin W (**1**) as 4,6-dihydroxy-2,3-dimethoxy-3-methyl-10-propyliden-2-azaspiro [4.5]dec-8-ene-1,7-dione. This structure was confirmed by the data of its ESI and HRESIMS, which showed the dimeric sodium form [2M+Na]+ and sodiated cluster [M+Na]+ at m/z 645 and 334.1275. When the ESIMS spectrum was recorded in negative mode, the pseudomolecular ion [M−H]− was observed at m/z 310.

The relative stereochemistry assigned to **1** and depicted in Scheme 1 was determined by the correlations observed in its NOESY spectrum (Table 2)[11] compared with the results of the same experiment carried out with triticone E (**6**).[6]

Table 1
^{1}H and ^{13}C NMR data and HMBC correlations for spirostaphylotrichin W (**1**) recorded in CDCl3[a,b]

Position	δ_C[c]	δ_H (J values in Hz)	HMBC
1	167.6 (C)	—	H-9, H-6, H-4
3	88.8 (C)	—	H-4, HO-4, MeO, Me-11
4	70.1 (CH)	4.12 d (11.5)	H-12, H-6, HO-4, Me-11
5	56.6 (C)	—	H-9, H-12, H-6, H-4, HO-6, Me-11
6	73.5 (CH)	4.75 d (1.7)	H-8, H-4, HO-6
7	196.8 (C)	—	H-9, H-6, HO-6
8	120.5 (CH)	5.94 d (9.9)	H-12
9	153.2 (CH)	7.11 d (9.9)	H-12, H2-13
10	128.9 (C)	—	H-9, H-8, H-6, H-4, H2-13
11	17.5 (CH3)	1.69 s	H-4
12	149.5 (CH)	6.12 t (7.4)	H-9, H2-13, Me-14
13	23.4 (CH2)	2.24 m	H-12, Me-14
		2.15 m	
14	13.2 (CH3)	1.05 t (7.4)	H-12, H2-13
15	64.5 (CH3)	3.94 s	—
OMe	52.7 (CH3)	3.51 s	Me-11
OH-6	—	3.80 d (1.7)	—
OH-4	—	2.66 d (11.5)	—

[a] The chemical shifts are in δ values (ppm) from TMS.
[b] 2D ^{1}H, ^{1}H (COSY) ^{13}C, ^{1}H (HSQC) NMR experiments delineated the correlations of all the protons and the corresponding carbons.
[c] Multiplicities were assigned by DEPT spectrum.

Table 2
NOESY data for spirostaphylotrichin W (**1**)

Irradiated	Observed
H-4	Me-11, HO-4
H-8	H-9
H-9	H-8, H-12
H-12	H_2-13, Me-14
Me-11	Me-15
HO-4	H-4, OMe, HO-6
HO-6	H-6, HO-4

In fact, the expected correlations between HO-6 and HO-4 with their geminal protons (H-6 and H-4) as well as those between H-12 with H_2-13 and Me-14 were observed. Furthermore, the significant correlation between H-4 and Me-11 and OH-4 and OMe were observed and allowed localization of both these groups in *cis* at C-4 and C-3, respectively. Similar correlations were also observed in triticone E (**6**). The only difference was the lack of the significant correlation observed in **6** between Me-11 and H-6, indicating that the stereochemistry at C-6 of **1** must be inverted. These results were confirmed by the significant coupling observed in **1** between HO-4 and HO-6, indicating that the relative stereochemistry of the chiral carbons of **1** was $3S^*,4S^*,5S^*$ and $6S^*$. Furthermore, the *Z*-stereochemistry of the double bond between C-8 and C-9 was deduced from the values for a *cis*-coupling as reported above for the corresponding protons. The same *Z*-stereochemistry of the double bond between C-10 and C-12 was determined by the NOESY correlation observed between H-12 and H-9. The relative stereochemistry assigned to **1** was also confirmed by an inspection of its Dreiding model, indicating that spirostaphylotrichin W was formulated as ($3S^*,4S^*,5S^*,6S^*,9Z$, $10Z$)-4,6-dihydroxy-2,3-dimethoxy-3-methyl-10-propyliden-2-azaspiro[4.5]dec-8-ene-1,7-dione. It appeared to be in diastereomeric relationship with spirostaphylotrichin V (**2**). The common names given to the spirostaphylotrichins have a somewhat complex history (Table 3).[13]

used, allowing first the isolation of the new spirostaphylotrichins E, F, G, H, I, K, L, M and S and then of spirostaphylotrichins N, O, P and T.[15,16] The spirostaphylotrichins A, C, D, R and Q were than isolated together with the new spirostaphylotrichins U and V from the fungus *Curvularia pallescens*.[7] Finally, the isolation and the biosynthetic studies on spirostaphylotrichins and related microbial metabolites were reviewed in 1996.[13] Here these compounds, including the new compound (**1**), are referred to as spirostaphylotrichins, except for **6**, which was never referred to as a spirostaphylotrichin in the literature and therefore has triticone E as its sole name.

Two different biological assays were performed with the isolated compounds as described in the experimental section. Compounds **3** and **4**, as well as **6** and **7**, were assayed as a mixture. In the cheatgrass coleoptile elongation assay at a concentration of 10^{-3} M, **5** was the most active compound, reducing 5-day coleoptile elongation to 33% of the control (Fig. 1A). **1**, **2** and the mixture **3**/**4** showed intermediate activity, while **6**/**7** did not significantly suppress coleoptile elongation relative to the control. In analysis of variance of the coleoptile elongation bioassay, all compounds except the **6**/**7** mixture significantly reduced coleoptile elongation relative to the control ($P<0.001$).

In the leaf puncture assays on young leaves of wheat (*Triticum durum* L.), tomato (*Lycopersicon esculentum* L.), sowthistle (*Sonchus arvensis* L.), and cheatgrass (*Bromus tectorum* L.) (Fig. 1B), the mixture of **3**/**4** showed by far the highest activity on all four plant species, while **5** showed moderate activity. **1**, **2** and the mixture of **6**/**7** showed no activity. These results are in agreement with those previously reported for the known compounds **5**, **3**/**4** and **6**/**7**,[6] and also for **2**.[7] In analysis of variance of the leaf puncture bioassay, both compound and plant species main effects were highly significant ($P<0.0001$), but only **5** and the mixture **3**/**4** caused leaf lesions that were significantly larger than the control (0 mm). Tomato was the most sensitive species and cheatgrass was the least sensitive.

Table 3
History of the common names given to the known compounds **2**–**7** produced by *P. semeniperda*

Compounds	Spirostaphylotrichins names	Source	References	Triticones names	Source	References
2	V	*Curvularia pallescens*	Abraham et al. 1995[7]			
3	A	*Staphylotrichum coccosporum*	Sandmeier and Tamm, 1989a[14]	C	*Pyrenophora tritici-repentis*	Hallock et al., 1993[6]
		C. pallescens	Abraham et al. 1995[7]			
4/5	C/D	*S. coccosporum*	Sandmeier and Tamm, 1989a[14]	A/B	*P. tritici-repentis*	Sugawara et al., 1988[8]
		C. pallescens	Abraham et al. 1995[7]			
7	R	*S. coccosporum*	Sandmeier and Tamm, 1989a[14]	F	*P. tritici-repentis*	Hallock et al., 1993[6]
		C. pallescens	Abraham et al. 1995[7]			
6				E	*P. tritici-repentis*	Hallock et al., 1993[6]

Spirostaphylotrichins C and D, the first two members of this class of natural compounds, were obtained as a mixture and named triticone A and B. Previously they were also obtained as phytotoxins from the tan spot pathogen of wheat, *Drechslera tritici-repentis* (=*Pyrenophora tritici-repentis*).[8] From the same fungus four related metabolites were obtained and named triticone C, D, E, and F.[6] These latter two compounds were isolated as a mixture.

At essentially the same time, spirostaphylotrichins A, B, C, D and R, together with F and Q were also isolated from the tropical fungus *Staphylotrichum coccosporum*.[14] Several of these compounds were identical to the triticones isolated from *P. tritici-repentis* (Table 3). The structures of these spirocyclic substances, with the unusual substituted γ-lactam moiety, prompted initiation of biosynthetic studies. To achieve this goal mutant strains of *S. coccosporum* were

To identify which structural features of these compounds are essential to account for these differences in biological activity would require preparation of derivatives by systematic chemical transformation of the functional groups present. **5**, and the mixture of **3**/**4** are active in both bioassays, whereas the remaining compounds show relatively low activity, suggesting that the presence of an exocyclic methylene group at C-3 is essential for the toxicity of these compounds. The results of the coleoptile elongation assay for **1** and **2** compared to those of the mixture **6**/**7** highlight how the presence of different group at C-3 can affect the toxic activity. The stereochemistry of the different chiral carbons is also likely to play a fundamental role in determining toxicity, but the presence of several of these compounds in mixture in the present study does not permit speculation as to the nature of this role. The high toxicity
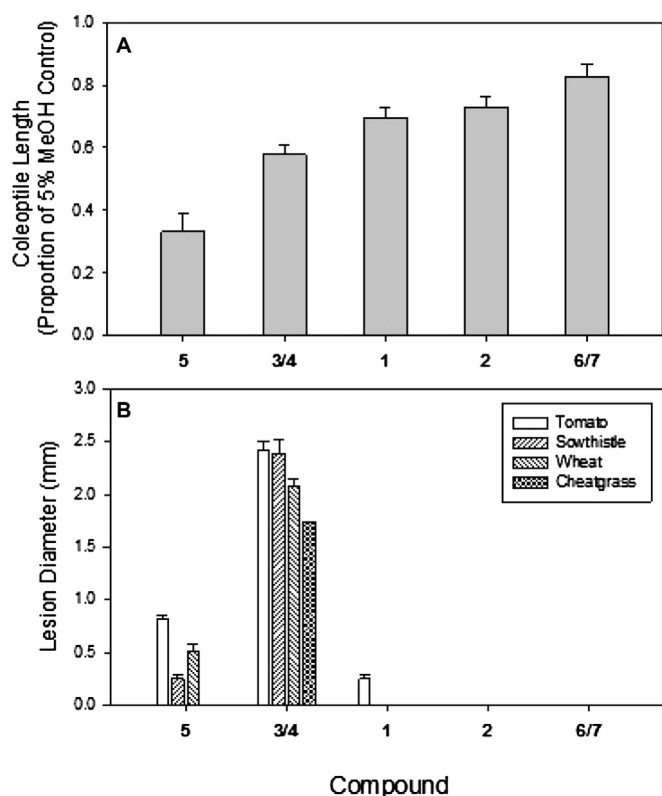
Fig. 1. Toxic activity of compounds **1–7** in (A) cheatgrass seedling coleoptile elongation bioassays and (B) leaf puncture bioassays on four plant species. Error bars represent standard error of the mean ($n=15$ for leaf puncture bioassay, $n=18$ for seedling bioassay).

of **5** relative to **3**/**4** in the seedling bioassay contrasted strongly with its considerably lower toxicity in the leaf puncture bioassay. It may be that the mixture **3**/**4** is more important in pathogenesis on leaves and therefore more important for *P. tritici-repentis*, a foliar pathogen, whereas **5**, which was previously reported as inactive in leaf puncture bioassays, is more likely to be directly involved with pathogenesis on seeds. Compound **5** has also been detected in solid wheat seed culture of *P. semeniperda* (Masi et al., unpublished data), supporting this idea.

In conclusion, this manuscript reports the isolation for the first time of the five well known spirostaphylotrichins A, C, D, R, V and also triticone E as metabolites of *Pyrenophora semeniperda*, as well as characterizing the new spirocyclic γ-lactam spirostaphylotrichin W.

3. Experimental

3.1. General experimental procedures

Optical rotation was measured in $CHCl_3$, unless otherwise noted, on a Jasco polarimeter; IR spectra were recorded as glassy films on a Perkin–Elmer Spectrum One FT-IR spectrometer and UV spectra were taken in MeOH solution (unless otherwise noted) on a Perkin–Elmer Lambda 25 UV/Vis spectrophotometer; ^1H and ^{13}C NMR spectra were recorded at 400 and 600 and at 100 and 125 MHz, respectively, in $CDCl_3$ on Bruker spectrometers. The same solvent was used as internal standard. DEPT, COSY-45, HSQC, HMBC and NOESY experiments[11] were performed using standard Bruker microprograms. HRESIMS spectra were recorded on Thermo LTQ Velos spectrometer; ESI and APCIMS spectra were recorded on Agilent Technologies 6120 Quadrupole LC/MS instrument. Analytical and preparative thin layer chromatography (TLC) were performed on silica gel (Merck, Kieselgel 60, F_{254}, 0.25 and 0.5 mm, respectively) plates. The spots were visualized by exposure to UV radiation (253), or by spraying first with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min. Column chromatography was performed on silica gel (Merck, Kieselgel 60, 0.063–0.200 mm).

3.2. Fungal strains

The strains used in this study were obtained from *Bromus tectorum* seed bank samples collected at the Whiterocks study site in northern Skull Valley, Tooele County, UT (WRK; −112.7780 long, 40.3282 lat, 1446 m elevation) and at Ten Mile Creek study site in Box Elder County, UT (TMC; −113.136 long, 41.8649 lat, 1453 m elevation). The field collections were made in November 2010. The anamorph (*Drechslera campanulata*) of the pathogen *P. semeniperda* forms macroscopically visible fruiting structures (stromata) that protrude from the surface of killed seeds in the seed bank. To obtain pure strains from field-collected killed seeds, individual stromata were surface-sterilized, wounded by breaking off the tip, and incubated in sterile water. New conidia produced at the wounded tip were then transferred to a small volume of sterile water using a needle, and the conidial suspension was poured over water agar. Excess water was decanted, and the plates were incubated for 8 h at room temperature. Single germinated conidia free of apparent contamination were then transferred using a needle under a dissecting microscope directly to MAM (modified alphacel medium) plates. We used the procedure of Campbell et al. (2003)[17] to stimulate maximum conidial production. Conidia were then harvested, tested for germinability, and stored dry at laboratory temperature in snap-cap vials.[2]

3.3. Production, extraction and purification of metabolites from *Pyrenophora semeniperda* strains

The strains TMC10-34 and WRK10-22 were grown in PDB (potato dextrose broth) culture at laboratory temperature by inoculating 500 mL of broth in 1 L Erlenmeyer flasks with 3 mg of dry conidia and incubating in shaker culture for 14 days. Mycelium was then removed from the medium by centrifugation and filtering, and the resulting filtrates were lyophilized and frozen at −20 °C until extraction and analysis.

The lyophilized culture filtrate of strain WRK10-22 (2 L) was dissolved in distilled H_2O (1/10 of its original volume, pH 4.3) and then extracted with EtOAc (3×500 mL). The organic extracts were combined, dehydrated (Na_2SO_4) and the solvent evaporated under reduced pressure, yielding a brown oil (330.5 mg). The organic extract was fractionated by column chromatography on silica gel, eluted with the $CHCl_3$/*i*-PrOH (95:5). Eight homogeneous fraction groups were collected. The residue (42.3 mg) of the first fraction group was further purified by TLC on silica gel, eluent $CHCl_3$/*i*-PrOH (95:5), yielding a mixture of **3** and **4**, which were produced in an approximately 1:1 ratio [3.8 mg, 1.9 mg/L, R_f 0.57, eluent $CHCl_3$/*i*-PrOH (95:5), R_f 0.66, eluent EtOAc/*n*-hexane (6:4)] and identified as spirostaphylotrichins C and D. The residue (35.5 mg) of the second fraction was further purified by TLC on silica gel, eluent $CHCl_3$/*i*-PrOH (95:5) yielding a homogeneous amorphous solid (**1**, 12.0 mg, 6.0 mg/L, R_f 0.32, eluent $CHCl_3$/*i*-PrOH (95:5), R_f 0.81, eluent EtOAc/*n*-hexane (6:4)) and being a new compound was named spirostaphylotrichin W. The residue (56.6 mg) of the third fraction was further purified by TLC on silica gel, eluent $CHCl_3$/*i*-PrOH (9:1) yielding a homogeneous amorphous solid (**2**, 4.4 mg, 2.2 mg/L, R_f 0.54, eluent $CHCl_3$/*i*-PrOH (9:1), R_f 0.23, eluent EtOAc/*n*-hexane (6:4)), which was identified as spirostaphylotrichin V. The residue (82.2 mg) of the fourth fraction group was further purified by TLC on silica gel, eluent $CHCl_3$/*i*-PrOH (9:1), yielding a mixture of **6** and

7, which were produced in an approximately 1:1 ratio (17.4 mg, 8.7 mg/L, R_f 0.20, eluent CHCl$_3$/*i*-PrOH (9:1), R_f 0.28, eluent EtOAc/*n*-hexane (6:4)) and identified as a mixture of triticone E and spirostaphylotrichin R.

The lyophilized culture filtrate of strain TMC10-34 (3 L), was dissolved in distilled H$_2$O (1/10 of its original volume, pH 4.3) and then extracted with EtOAc (3×500 mL). The organic extracts were combined, dehydrated with Na$_2$SO$_4$ and the solvent evaporated under reduced pressure, yielding a brown oil (298.2 mg). The organic extract was fractionated by column chromatography on silica gel, eluted with the CHCl$_3$/*i*-PrOH (95:5). Five homogeneous fraction groups were collected. The residue (48.2 mg) of the third fraction was further purified by TLC on silica gel, eluent CHCl$_3$/*i*-PrOH (9:1) yielding a homogeneous amorphous solid (**5**, 12 mg, 4.0 mg/L, R_f 0.70, eluent CHCl$_3$/*i*-PrOH (9:1), R_f 0.34, eluent EtOAc/*n*-hexane (1:1)), which was identified as spirostaphylotrichin A.

*3.3.1. Spirostaphylotrichin W (**1**).* Amorphous solid, $[\alpha]_D^{25}$ −76 (*c* 0.24); λ_{max} nm (log ε) 290 (5.02), 202 (5.11); ν_{max} 3448, 2942, 1716, 1681, 1617, 1587, 1560, 1438, 1405, 1382, 1234 cm^{-1}; ^{1}H and ^{13}C NMR: Table 1; HRMS (ESI+): 2M+Na$^+$, 645; M+Na$^+$, found 334.1275. C$_{15}$H$_{21}$NNaO$_6$ requires 334.1267; ESIMS (−): M−H$^-$, 310.

*3.3.2. Spirostaphylotrichin V (**2**).* Amorphous solid, ^{1}H NMR was very similar to that previously reported.[7] ESIMS (+): 2M+Na$^+$, 645, M+Na$^+$, 334; ESIMS (−): M−H$^-$, 310.

*3.3.3. Spirostaphylotrichins C and D (**3** and **4**).* Amorphous solid, ^{1}H NMR was very similar to that previously reported.[6] ESIMS (+): M+Na$^+$, 300; ESIMS (−): M−H$^-$, 276, APCI (+): M+H$^+$, 278.

*3.3.4. Spirostaphylotrichin A (**5**).* Amorphous solid, $[\alpha]_D^{25}$ +3.4 (*c* 1.0, CHCl$_3$); λ_{max} nm (MeCN) (log ε) 285 (3.80), 218 (4.29); ν_{max} 3417, 2941, 1722, 1683, 1666, 1620, 1556, 1440, 1371, 1261, 1107 cm^{-1} [lit. 6: $[\alpha]_D^{25}$ +2.0° (*c* 1.0, CHCl$_3$); UV λ_{max} (EtOH) nm (log ε) 228 (3.92), 222 (4.04); IR ν_{max} (film) cm^{-1} 3450, 2900, 1720, 1685, 1650, 1620, 1580, 1440, 1370, 1260, 1100]; ^{1}H NMR was very similar to that previously reported;[6] HRMS (ESI+): 2M+Na$^+$, found 581.2104. C$_{28}$H$_{34}$N$_2$NaO$_{10}$ requires 581.2112, M+Na$^+$, found 302.0994. C$_{14}$H$_{17}$NNaO$_5$ requires 302.0982, M+H$^+$, found 280.1176. C$_{14}$H$_{18}$NO$_5$ requires 280.1184.

*3.3.5. Triticone E and spirostaphylotrichin R (**6** and **7**).* Amorphous solid, ^{1}H NMR was very similar to that previously reported;[6,7] ESIMS (+): 2M+Na$^+$, 617, M+Na$^+$; 320; ESIMS (−):*z* [M−H]$^-$, 296.

3.4. Bioassays methods

3.4.1. Cheatgrass coleoptile elongation assay. Compounds **1**, **2**, **5** and the mixtures of compound **3**, **4** and **6**, **7** were first dissolved in MeOH, and then brought up to the assay concentration of 10^{-3} M with distilled water (the final content of MeOH was 5%). One ml of the solution for each sample and concentration was pipetted into each of three 6 cm Petri dishes onto the surface of one filter paper. Six host seeds were arranged onto the surface of each filter paper in a pattern that made it possible to track individual seeds. Petri dishes were sealed with parafilm to retard moisture loss, stacked in plastic bags, and incubated at 20 °C with a 12:12 h photoperiod. Germination was scored each day, and germination day was tracked individually for each seed. Five days after germination, the coleoptile length of each seedling was measured and recorded using electronic calipers. Most seeds germinated within three days. Seeds that did not germinate (<5%) were excluded from analysis, while seeds that produced a radicle but no coleoptile were scored with a coleoptile length of zero. Coleoptile length data were log-transformed to improve homogeneity of variance prior to analysis of variance. Seeds were incubated in 5% MeOH in the control treatment.

3.4.2. Leaf puncture assay. Young leaves of wheat (*Triticum durum* L.), tomato (*Lycopersicon esculentum* L.), sowthistle (*Sonchus arvensis* L.), and cheatgrass (*Bromus tectorum* L.) were used for this assay. Compounds **1**, **2**, **5** and the mixtures of compound **3**, **4** and **6**, **7** were dissolved in MeOH and then brought up to the assay concentration of 10^{-3} M with distilled water (the final content of MeOH was 5%). One cm sections of leaf (grasses) or 8 mm leaf disks cut with a cork borer (dicots) were placed in Petri dishes (five leaves for each species in each of three replicates) on the surface of a water-saturated filter paper. The test solutions (15 µL) were applied on the axial side of leaves that had previously been needle-punctured. Droplets (15 µL) of MeOH in distilled water (5%) were applied on leaves as a control. The dishes were sealed with parafilm and incubated at 20 °C with a 12:12 h photoperiod. Leaves were observed daily and scored for symptoms after 4 days. The effect of toxins on the leaves was evident as necrotic spots or lesions surrounding the puncture. Maximum lesion diameter for each leaf puncture was measured using electronic calipers.

Acknowledgements

Supplementary data

^{1}H and ^{13}C NMR data as well as COSY, HSQC, HMBC, NOESY, ESIMS, IR and UV spectra of Spirostaphylotrichin W (**1**). Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tet.2013.12.056.

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